Morphological Studies in the Life-Histories of Bacteria.

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(Plates 16–20.)

It has of course long been recognised that changes in the cultural environment of bacteria may sometimes be followed by corresponding morphological adaptations. By many students such changes in form have been summarily dismissed on the facile theory that cultural contamination has occurred. The rigid precautions taken in these experiments to exclude such accident, and the fact that gemmation took place under observation on the warm stage, dispose of the contamination theory.

By other students aberrant morphological types of bacteria are frequently put aside on account of their supposed involutionary nature. The term "involution form" may perhaps be legitimately applied to the bizarre deformities seen in dying or dead individuals in old, or otherwise unsuitable, media. But to apply the term to young, freely growing, freely dividing organisms under the optimum cultural conditions employed throughout in these experiments would be hardly reasonable.

By still other observers the morphological changes which may follow alteration of bacterial environment have in the past been looked upon as genuine mutation phenomena, and by some authorities the term polymorphic, or pleomorphic, is inaccurately restricted to such alleged examples of mutation. These latter writers deny the occurrence of bacterial mutation, and dogmatically assert that pleomorphism—in the limited sense above defined—is unknown amongst the bacteria, heedless of the fact that the genuine type of pleomorphism exhibited, for example, by the protozoa and the parasitic fungi in the orderly sequence of the manifold cycles of their complex life-histories, would equally apply to the bacteria, once it was proved that they too can reproduce themselves in other ways than by simple transverse binary fission into equal parts.

Finally, it is often assumed that, because bacteria appear to breed true to type—that is, to laboratory type—in standardised laboratory cultures, simple transverse binary fission is the sole method of reproduction under natural saprophytic and parasitic conditions which are not, and never can be, standardised.

As, however, will appear, evidence of complex bacterial life-cycles is constantly before us, even in ordinary standardised laboratory media, though it may, and often does, require persistent looking for.

The truth, in fact, appears to be that we have gone astray in this matter, because we have in the past invoked too easily the theories of contamination, of involution forms, and of mutation,* and have forgotten that the natural environment of bacteria, whether as saprophytes or as agents of disease, is in a perpetual state of flux.

From the morphological observations here recorded, it is clear that bacteria can, and do, reproduce themselves in other ways than by simple binary fission alone, and that the life-cycle in some cases includes an invisible, or almost invisible phase. Our present conception, therefore, of the *rôle* played by bacteria, both as saprophytes and as causal agents of disease and its sequels, will have to be profoundly modified.

According to current bacteriological theory the "lower" bacteria, whether living a parasitic or a saprophytic existence, are unicellular organisms which are only capable—apart, in certain cases, from endosporulation of a special type—of reproducing themselves by a simple process of transverse binary fission into two equal parts.

In previous publications (1) of studies of the life-history of bacteria, I have brought forward evidence which strongly suggested, if it did no more, that the life-cycle of certain of the "lower" bacteria is one of great complexity. From this evidence the conclusion was difficult to escape that under parasitic conditions simple binary fission is probably only one of many phases in the bacterial cycle, which includes an invisible, or almost

* As already stated, the observations here recorded are confined to cultures from single primary colonies or from single individuals, and in consequence these observations only deal with changes in morphology which are not associated with observed changes in cultural, fermentative and serological reactions. I have, however, noted, especially in the case of the *B. Shiga-Kruse* and of the *B. paratyphosus B*, that profound cultural, "biochemical," and even serological, changes may take place in the case of secondary colonies from pure cultures in some of the plates after prolonged standing and repeated subculturing from acid media. Changes in fermentation reactions of secondary colonies of bacteria have in the past been frequently noted by numerous observers, and the occurrence of a genuine form of bacterial mutation has been invoked to explain these changes. In many such cases the occurrence of morphological types of aberrancy has also been noted, and again the mutation theory has been used in explanation thereof. The most striking of these are the observations of Horrocks (5) in 1911 in the case of the *B. typhosus*, independently confirmed by Almquist in the present year. In all these observations, however, of changes in morphology, whether associated or not with fermentation and serological changes, the question of such changes representing phases in bacterial life-histories in orderly sequence does not appear till now to have been raised. And this appears to be due to the fact that the morphological changes have not been studied by continuous observation of growth from single organisms on the warm stage, which I have shown to be essential to correct interpretation of alleged mutation phenomena, whether of a morphological or of a "biochemical" nature.

invisible, filterable stage, and that this also applies to laboratory cultures of organisms of certain diseases. In passing, I may note that my observations in 1914 in typhus fever, and, later, in the same disease,* in cerebrospinal fever, in scarlet fever and in measles, as to the existence in the infected body fluids of filterable infective viruses, and of growth from these of non-filterable bacteria, has since been confirmed in the case of laboratory cultures of the azotobacteria by Löhnis (2), in the summer of 1916. This observer, however, has recorded no details of his filtration experiments, and does not state if his cultures were from single colonies, or from single organisms, or if he carried out direct observations of growth from single individuals on the warm stage.

In the present communication I propose to present further pictorial evidence of the complicated life-history of the enteric group of bacteria in so far as this can be studied in laboratory media as opposed to the more natural *milieu* of infected tissues and body fluids. And to morphological studies of the members of the enteric group I have also added observations on a single strain of a coliform bacillus.

The strains of organism of the enteric group examined are as follows, their source being also indicated.

B. typhosus	4 strains : Strain 1, Lister Institute ; Strains 2, 3, 4, Carrier strains, Addington.
B. paratyphosus A	3 strains : Strain 1, Lister Institute ; Strains 2, 3, Carrier strains, Addington.
B. paratyphosus B	5 strains : Strain 1, Lister Institute ; Strains 2, 3, 4, 5, Carrier strains, Addington.
B. Shiga-Kruse	1 strain : Lister Institute.
B. Y of Hiss	5 strains : Strain 1, Lister Institute ; Strains 2, 3, 4, 5, Carrier strains, Addington.
B. Flexner	3 strains : Strain 1, Lister Institute ; Strains 2 and 3, Carrier strains, Addington.

In figs. 1–4 and Plates 16–19 will be seen the results obtained by study of dried film preparations from young cultures from single colonies, whilst in Plate 20, A, B, C, will be seen growth from single individuals studied on the warm stage.

All the strains of organisms shown were obtained from the Lister Institute, except the coliform organism.

It is not possible to reproduce here evidence that the morphological results noted in the case of all the strains enumerated above are identical with the

* Microphotographic evidence of growth from filterable virus to non-filterable bacteria in typhus fever, together with experimental evidence of pathogenicity at each stage, as well as evidence of complexity of life-history of the enteric organisms, was presented (3) to the Royal Microscopical Society in November, 1916.

results shown in the drawings and photographs of the selected strains. I must be content, therefore, with stating that the essential results were the same in all cases, approximately 1000 films having been examined. The organisms which I have chosen in order to illustrate my points here are the *B. typhosus* of Eberth, the *B. dysenteriæ* of Shiga-Kruse, the *B. dysenteriæ Y.* of Hiss, and a bacillus of the coliform group.

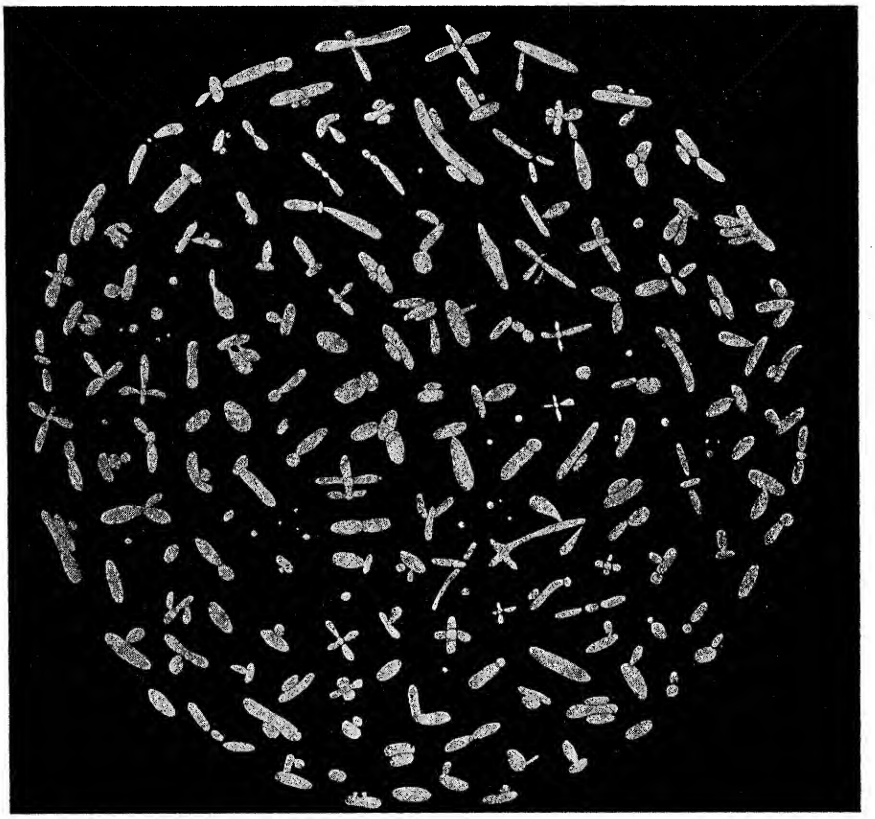

FIG. 1.—*B. typhosus.* +60 to phen. broth culture mixed with +10 to phen. broth culture from same.

In order to reduce the chances of error to the minimum I have, in addition to rigid precautions against contamination to be described later, submitted each strain of the organisms of the enteric group to searching identification tests, cultural, biochemical, and serological, both at the beginning, during the course of, and at the end of each set of observations, the additional precaution being taken of frequent replating on MacKonkey's medium and on agar, and of repeatedly restarting the whole process of examination by subculture from fresh single non-lactose-fermenting colonies on the former medium.

In every case the cultures under examination successfully passed at every stage the necessary standard identification tests, the final agglutination results of the three strains of members of the enteric group selected for demonstration being detailed below.

Shiga-Kruse Strain.—Final culture in broth, +10 phen. direct from broth, +20 phen. Dilution of antiserum 1/10, titre 1/1500, date of tubing 20.9.16 (Lister Institute).

Final dilutions ...	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560.	Control.
Result	Agg.	Agg.	Agg.	Agg.	Agg.	Agg.	Nil.	Nil.

Incubated for two hours at 56° C., and read after 24 hours at room temperature.

B. Typhosus Strain.—Final culture in broth, +10 phen. direct from broth, +60 phen.

Dilution of antiserum 1/20, titre 1/6000, date of tubing 31.3.16 (Lister Institute).

Final dilutions ...	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Control.
Result	Agg.	Agg.	Agg.	Agg.	Agg.	Agg.	Agg.	Nil.

Incubated for two hours at 56° C., and read after one hour at room temperature.

B. "Y" of Hiss.—Final culture in broth, +10 phen. direct from broth, +20 phen.

Dilution of antiserum 1/10, titre 1/1500.

Final dilutions ...	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560.	Control.
Result	Agg.	Agg.	Agg.	Agg.	Agg.	Agg.	Nil.	Nil.

Incubated for two hours at 56° C., and read after 24 hours at room temperature.

The precautions taken against contamination are as follows, the culture media employed throughout being peptone agar and peptone broth, the latter +10 to phenolphthalein, +20 to phenolphthalein, and +60 to phenolphthalein, the last being only exceptionally employed.

1. The acid broth in each case was, after tubing, autoclaved for 30 minutes at 120° C. under a pressure of 20 lb.

2. Control tubes of uninoculated acid broth were incubated at 37° C. for the same length of time as the inoculated tubes. In no case was any turbidity or deposit observed after prolonged incubation.

3. In many of the cases the acid broth was contained in specially made silica glass flasks, the narrow necks of which were closed with rubber teats sterilised by one hour's immersion in pure lysol, and subsequently dried in sterile metal boxes for 24 hours at 56° C. It this way it was possible

entirely to avoid the use of plugs of wool, and to inoculate, or withdraw fluid from, the tubes with sterile Pasteur pipettes in the actual flame of a Bunsen burner. The use of these flasks also gave an absolute guarantee that only clean vessels were employed, each flask being heated to not less than 300° C. before use.

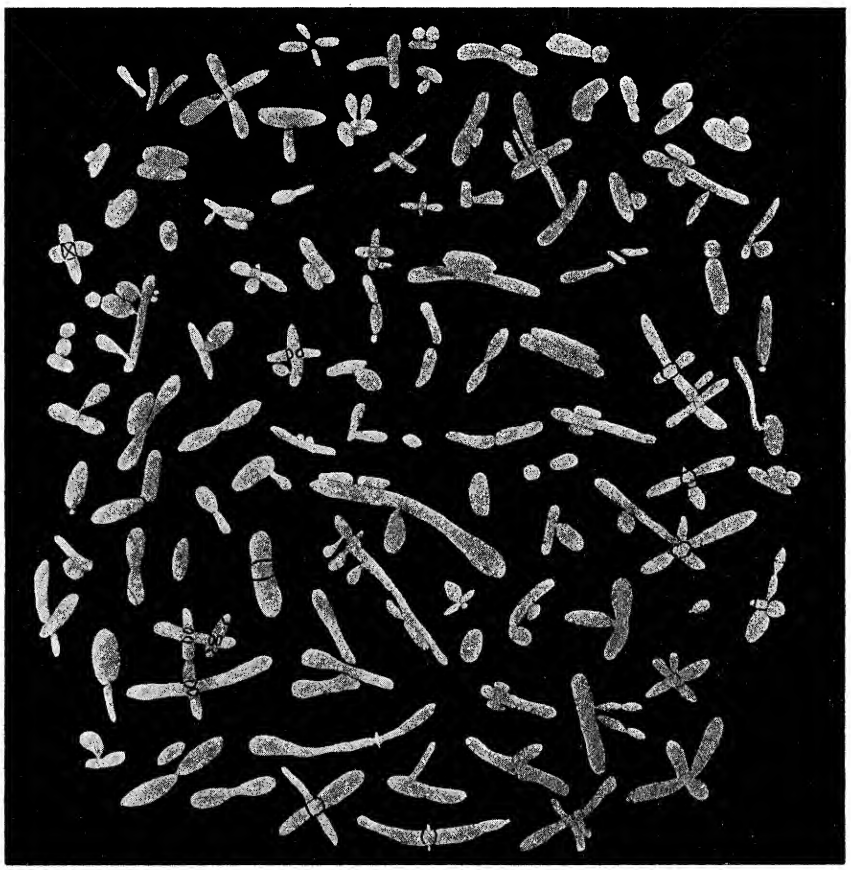

FIG. 2.—*B. coli communis.* Acid broth +20 to phen. mixed with broth sub-culture from same.

4. Deposits were obtained in all cases by centrifuging for three to five minutes the broth in small pointed serum tubes, each tube being sufficiently heated before filling to ensure carbonisation, and subsequent destruction of, any material left after routine cleaning by previous use. In this way it was possible to be certain that no organisms foreign to the inoculated broth under observation were present in the tubes employed.

5. All glass slides for microscopical examination were treated in the same

way, even after thorough cleansing with boiling acid and bichromate solutions. New slides were employed throughout.

6. The Congo-red emulsion* was made up daily, or on alternate days, with freshly distilled water, this being obtained for each set of experiments by distilling from a clean Jena flask fitted with new glass tubing. The

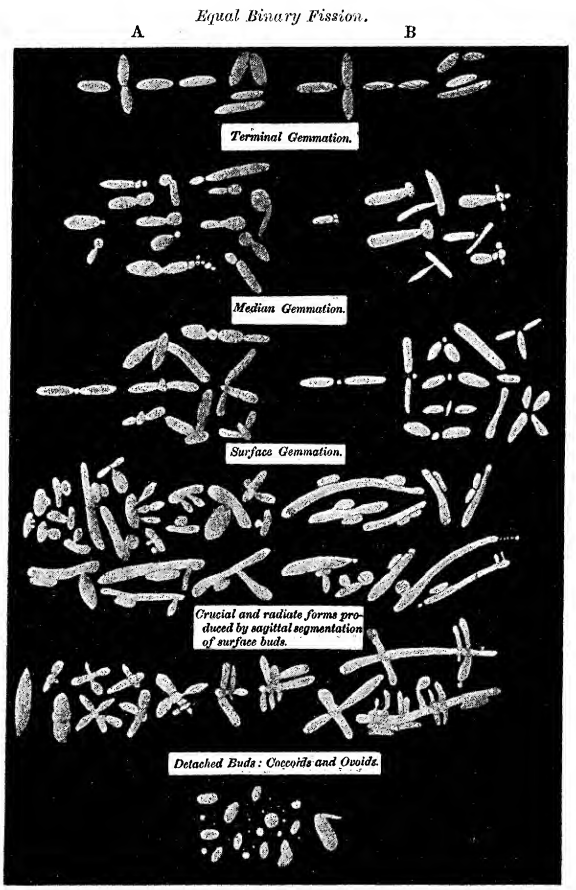

FIG. 3.—*B. Shiga-Kruse.* Acid broth +10 to phen. mixed with broth sub-culture from same.

emulsion was finally boiled in each case before use. In control films of Congo-red alone no organisms could be seen. In this way a dangerous source of error, due to mixing with tap water, or with distilled water from the ordinary laboratory still, was entirely avoided.

* In the case of dried films I employ a 1 per cent. solution of HCl in alcohol in order to avoid distortion of outline, control observations without the acid-alcohol bath showing that, if heat be not employed in drying, the use of a bath of this strength does not cause shrinkage or other distortion.

At the outset of the work, considerable difficulty was encountered in making satisfactory morphological studies of the organisms in question, on account of their small size, ordinary cultures of members of the enteric group providing organisms varying in size from approximately 0·5 μ to 2 μ. In Benians' Congo-red adsorption method, described by him in 1916 (4), I found, however, an invaluable method for studying the morphology of killed organisms, without any of the disadvantages inseparable from the use of basic stains, though in all cases the results obtained by the

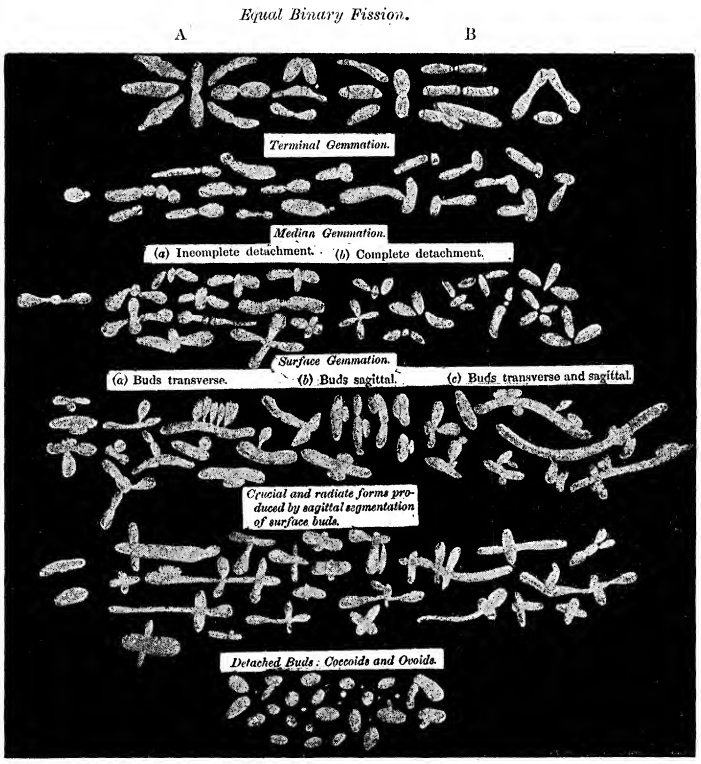

FIG. 4.—*B. Y of Hiss.* Acid broth + 20 to phen. mixed with broth sub-culture from same.

Congo-red method were confirmed by staining methods as well. The chief advantage of the method is the apparent increase in size of bacteria when emulsified with Congo red, as compared with the results obtained by staining methods, or even by Burri's adsorption method.

This apparent increase in size, however, was not sufficient for my purpose, as, although evidence of complex life-histories can—once one's attention has been arrested thereby—be unmistakably made out, both in stained films and in Congo-red films of ordinary cultures in + 10 broth, the size of the organisms

in these cultures is not sufficiently great to enable one to arrive with certainty at a correct interpretation of the different forms seen.

By the use, however, of broth +20 to phenolphthalein, and by sub-culture from this to agar or to MacKonkey's medium, thence back again to ordinary broth, +10 to phenolphthalein, I found that a great increase in size can with patience be obtained, still giving the classical cultural, fermentative, and serological reactions. This was particularly the case with the *B. typhosus*, with the *B. Shiga-Kruse*, with the *B. Y* of Hiss, and with the coliform organism examined. In the case of both the Paratyphoids, and of the *B. dysenteriæ* of Flexner, a considerable increase in size was also obtained by using these acid cultures, though so far I have not succeeded in obtaining the same increase as in the case of the other organisms mentioned.

Before going through the drawings, for which I am mainly responsible, and the photographs, for which Mr. Martin Duncan's skill and tireless enthusiasm are solely responsible, it is necessary briefly to deal with the possibility that many of the types of organism shown are merely involution forms.

That this is not the case is shown by the following considerations:—

1. The "aberrant" forms shown are young, freely growing, freely dividing organisms. By the use of the Congo-red method, the relative age of organisms can be fairly accurately gauged, owing to the fact that young organisms are brightly refringent, older organisms being faint or dark in colour.

2. In the case of the *B. typhosus*, the *B. Y* of Hiss, the *B.* of Shiga-Kruse, and the coliform organism, the growth in broth +20 to phenolphthalein was no less free and vigorous than in broth +10 to phenolphthalein, and it was in these that the largest and most "aberrant" types of organism occurred in the greatest numbers. Moreover, in broth +60 to phenolphthalein the degree of turbidity produced by the *B. typhosus* and by the organism of the coliform group was greatly in excess of that produced by these organisms in standard broth, +10 to phenolphthalein.

3. These "aberrant" types maintained their "aberrancy" for several sub-cultures when put back into broth +10 to phenolphthalein, even when the latter was inoculated direct with single colonies from MacKonkey's medium.

4. They were seen in small numbers, if carefully searched for, in ordinary cultures in broth, +10 to phenolphthalein, which had never been inoculated from broth of a higher acid titre.

This was also true of single colonies on MacKonkey's medium, or on agar, that had not at any time been derived from incubated broth cultures, but had been isolated direct from the fæces or urine of carriers.

5. The same types of "aberrancy" were seen in every one of the different organisms shown, as well as in all the strains of all the organisms not shown, and of the different strains of the organisms, single strains of which are shown.

In the accompanying photographs (Plates 16–19) and drawings attention is called to the following points :—

1. It is not claimed that a complete history of bacterial life-cycles can be worked out in acid broth cultures. This can only be obtained by extensive experimental observations, side by side with comprehensive morphological studies of organisms as they occur in the infected tissues and body fluids of subjects of disease. These, in the case of members of the enteric group, have yet to be undertaken.

2. In all the broth cultures studied, reproduction by simple binary fission was still the predominant feature, and in studying the "aberrant" types of reproduction of single living organisms on the warm stage on solid media, such as gelatin-agar, ordinary binary transverse fission was found eventually to hold the field mainly, though not absolutely, to the exclusion of other types of reproduction. It appears from these observations from single living individuals on the warm stage that reproduction by gemmation occurs freely, in conjunction with ordinary binary fission, only so long as growth proceeds in the thin layer of broth on the cover-slip, and largely comes to an end when colonies are beginning to form on the solid medium. This abrupt transition is well seen in Plate 20, as is also the familiar "slipping" phenomenon described by Hill in 1904.

3. The percentage population of "aberrant" forms in ordinary broth cultures, +10 to phenolphthalein, was low, but the chief types of "aberrancy" recorded could always be found if persistently searched for.

4. The percentage population of "aberrant" forms in broth cultures +20 and +60 to phenolphthalein, as well as in sub-cultures of these in ordinary broth cultures, +10 to phenolphthalein, was high, each field of the microscope yielding, in good films, characteristic types.

5. Each photograph is designed to show at the optimum focus not more than a very small number of types.

6. The number of types which can be seen in any acid culture exceeded 100. To reproduce a comprehensive picture by photographs alone was therefore impracticable.

7. In consequence it became necessary to make composite drawings with the camera lucida of the chief types observed in one or, at the most, two film preparations from one strain. These drawings represent, in the case of killed organisms, selected individuals from a large number of fields, and must therefore not be read as representing average fields.

8. At first sight inspection of the drawings in figs. 1 and 2, and of the photographs of killed organisms, suggests meaningless chaos.

9. Once, however, it is grasped that reproduction by gemmation is the key to the "aberrant" forms shown, and that gemmation may be terminal, median or superficial, the main types fall into line.

That true gemmation occurs of these three types is shown in Plate 20, representing growth from single organisms on the warm stage.

10. There is no evidence that a given strain represents a mixture of several strains, this suggestion being largely excluded by study of gemmating forms before fission has taken place, and by study of the actual process of gemmation on the warm stage.

11. The correct explanation of the superficial gemmation origin of the crucial and radiate forms shown is more difficult to establish than is that of the terminal, median and simple superficial forms of gemmation.

The points against a mere apposition explanation of these crucial and radiate forms are as follows:—

(*a*) Strict rectangular symmetry is the rule.

(*b*) The diameter of the central, brightly refringent node is frequently twice that of the organism which might otherwise be interpreted as lying in contiguity.

(*c*) Superficial buds can frequently be observed on the parent bacillary stem before "sprouting" has commenced, and during the act of sprouting.

12. Sagittal segmentation of buds can frequently be seen, both in the case of dried organisms and of single living organisms, before separation from the parent stem has begun. This sagittal segmentation can be seen in the terminal, median, and superficial buds.

13. Transverse segmentation of buds — ordinary binary fission — also frequently occurs, the parent stem also presenting buds undergoing sagittal segmentation, the actual occurrence of which was watched on the warm stage, as shown in Plate 20.

14. Undetached buds may vary in size from about 0·1 μ to several μ in their greater diameter, every intermediate size—from the filterable to the non-filterable—being capable of recognition in the same film in favourable cases (*vide* photographs of dried films).

15. The appearance of minute buds on a large scale is inconstant in broth cultures, as observed in dried films. In the study of growth from single organisms on the warm stage it occurs frequently, only a relatively small

number, however, coming to maturity on solid media, the majority fading and disappearing.

16. The appearance of the very minute forms, seen in figs. 1, 3, and 4, and Plates 16 and 20, makes it impossible to be certain, without prolonged observation on the warm stage, that, in attempting to obtain cultures from single individuals of normal size by Barber's method, or by the fragmented slip method of isolation, one is not in reality cultivating from several individuals. Unless therefore the presence of these minute forms can be excluded, the use of these two methods for obtaining cultures in liquid media from single organisms cannot be relied on.

17. The presence of these minute forms is probably the explanation of the apparent filterability through Chamberland filters of such relatively large organisms as the *Bacillus bronchisepticus*, and is perhaps responsible for the general view that even well-made Berkefeld filters are not suitable for bacteriological work.

18. By the dark-ground method of illumination many of these small detached buds appear as minute bacilli in the act of undergoing binary fission. Not infrequently these appear as coccoid bodies, if binary fission has not begun. In the study of aberrant bacterial forms with dark-ground illumination the use of the hanging drop method, apart from the inherent fallacies of dark-ground work, is fatal to correct interpretation unless streaming movements have first been reduced to a minimum. For example, it is frequently stated that apparent branching in bacteria is, in reality, simulated by mere apposition, and that observation of a hanging drop with dark-ground illumination will soon dispel the illusion, separation of apposed organisms sooner or later always taking place. On casual inspection of dark-ground hanging drops this statement appears to represent the truth, especially if streaming movements are still free. If, however, a drop of emulsion be firmly pressed under a cover-slip and then examined, it will be found, streaming movements now being reduced to a minimum, that detachment does not invariably take place. That this is not the result of pressure is shown by the fact that in favourable cases long lateral buds will exhibit wide lateral movements, whilst the base, or point of attachment to the parent stem, remains fixed. In other cases short lateral buds retain their relative position to the parent stem, itself exhibiting unfettered rotatory movements. The accuracy of these observations is confirmed in Plate 20 of warm stage studies.

In concluding it is perhaps unnecessary to point out that no claim whatever is here made that the complete life-histories of the bacteria of the

enteric group have been worked out. On the contrary the sole aim has been to show that simple binary fission is not the only method of reproduction of these organisms, and that only a fraction of what appears to be a highly complex life-cycle can be studied by cultivation in, or on, synthetic media.

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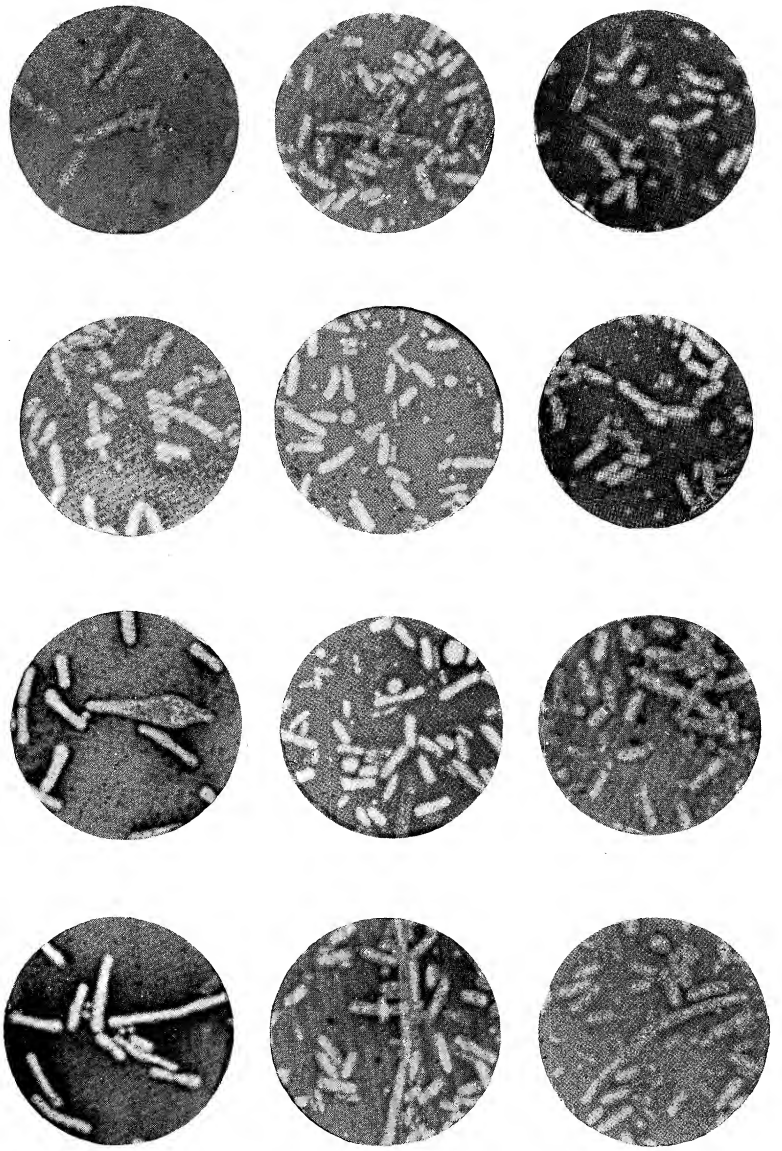

B. Shiga-Kruse.
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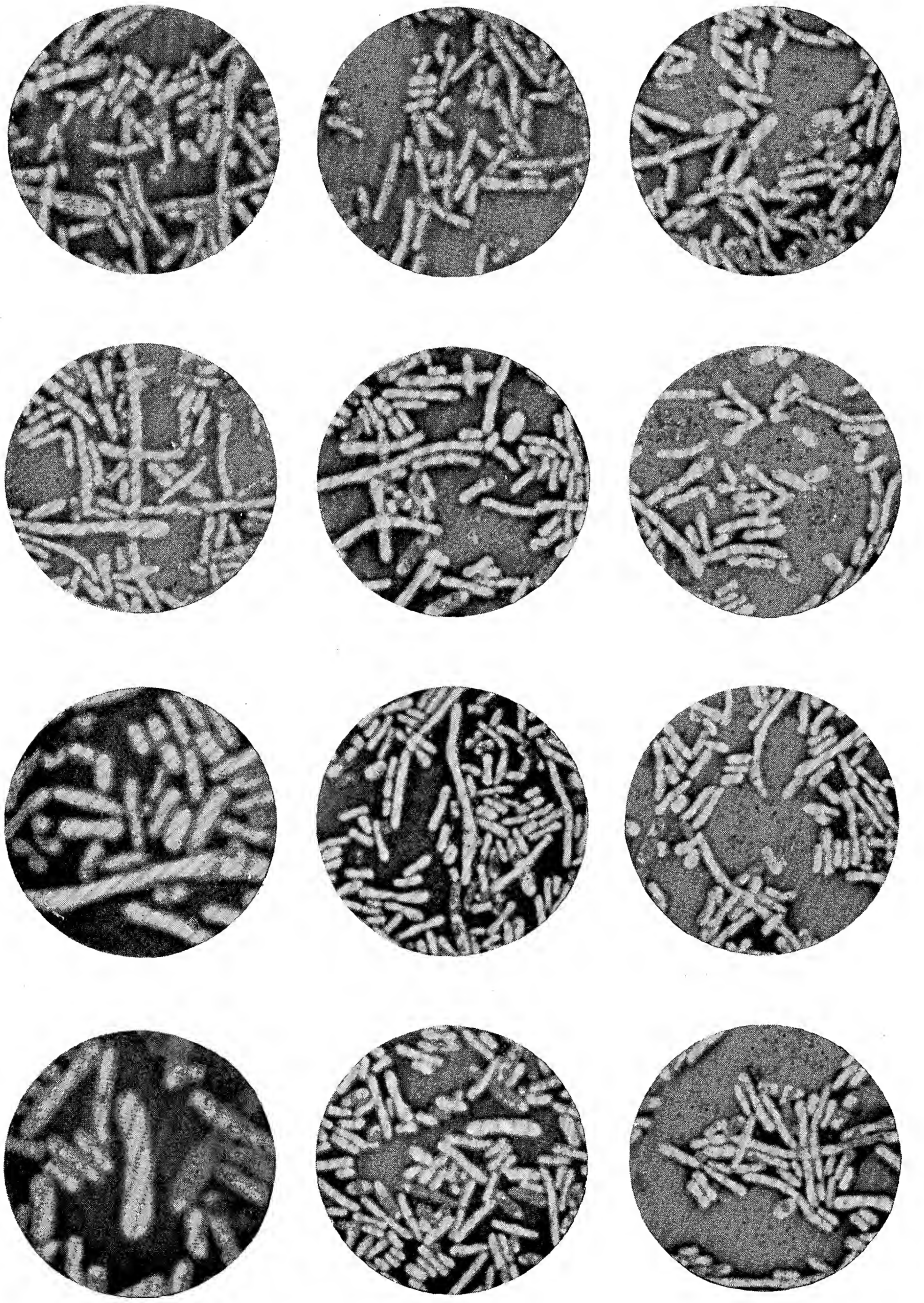

B. Coli Communis.
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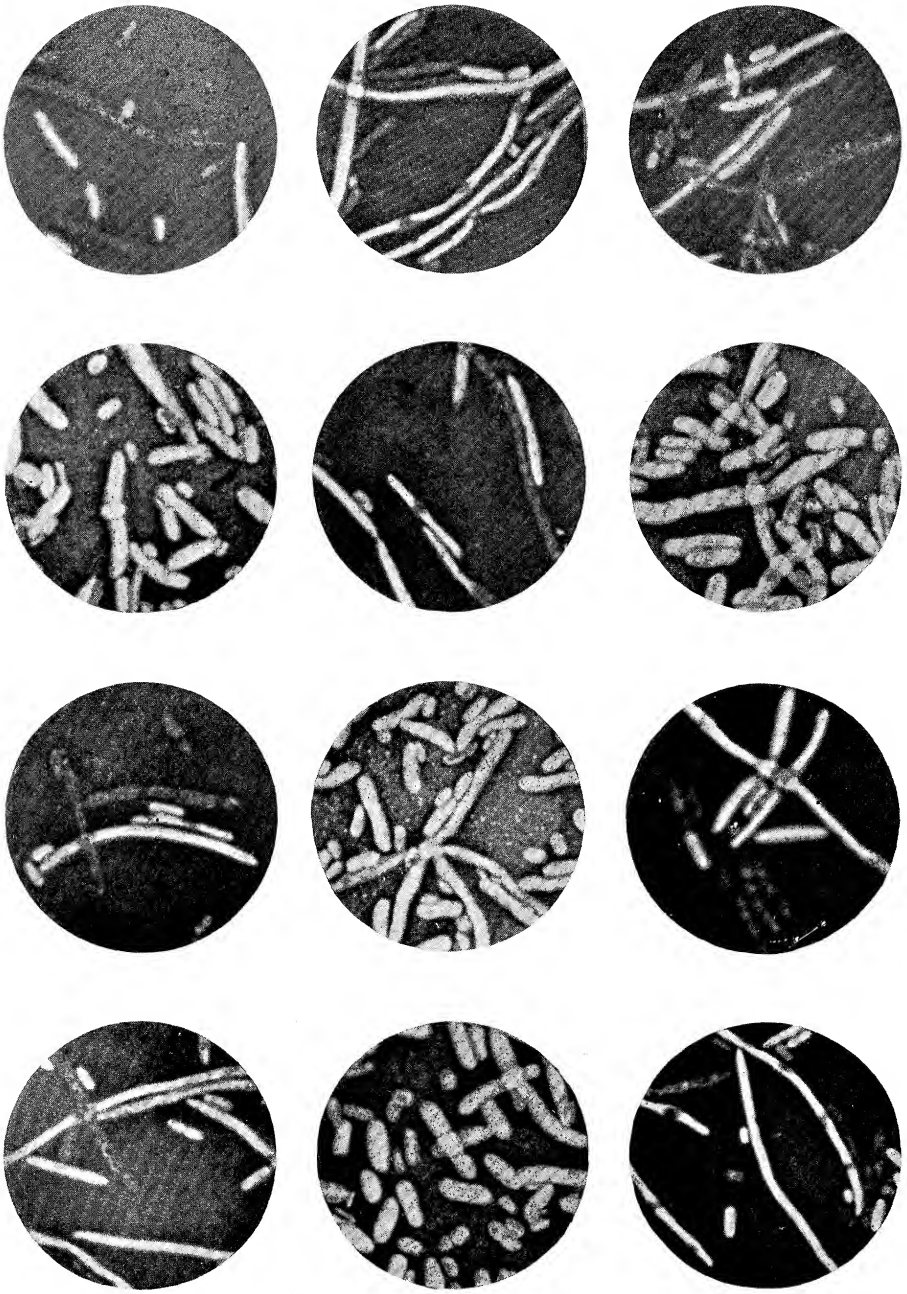

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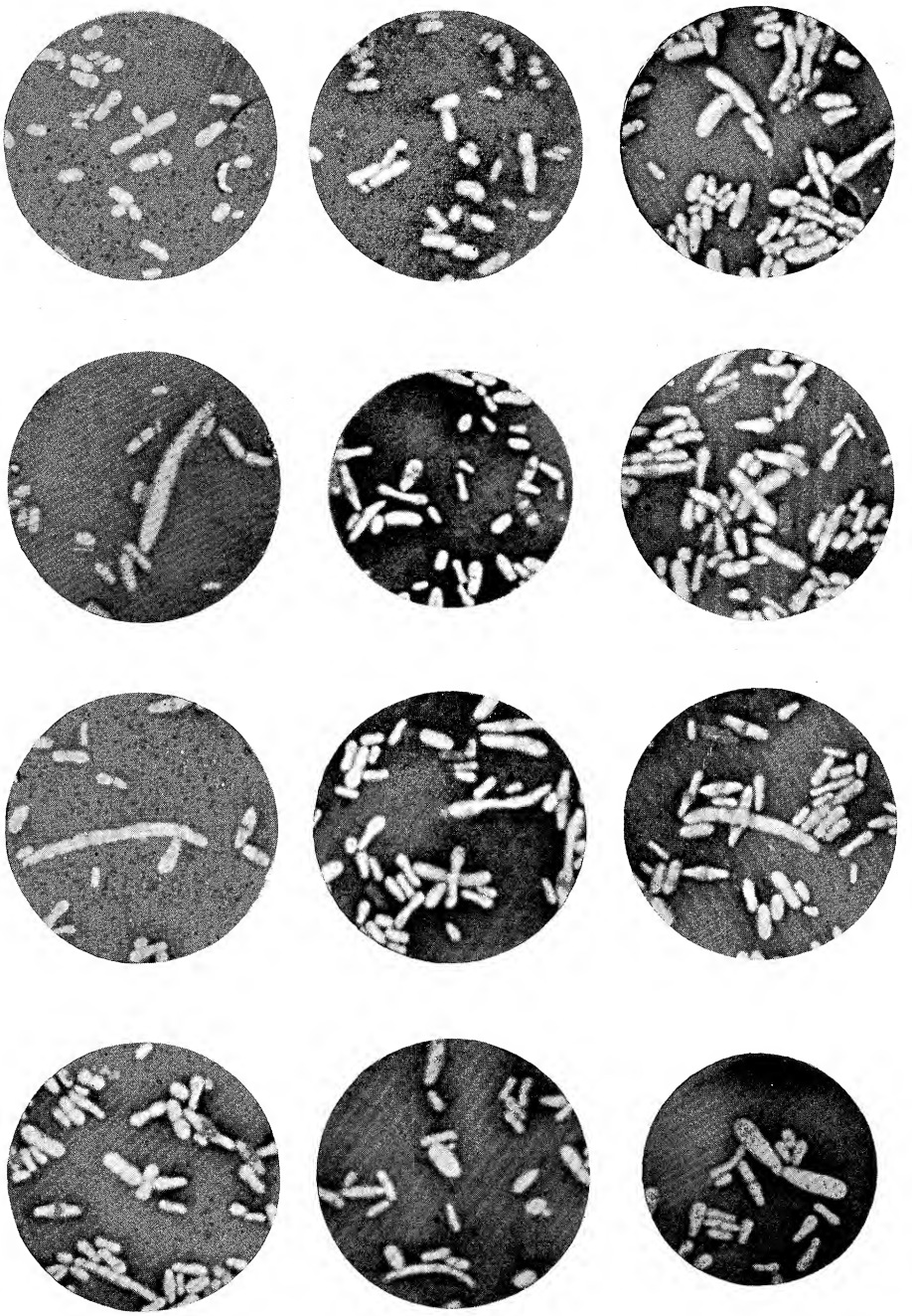

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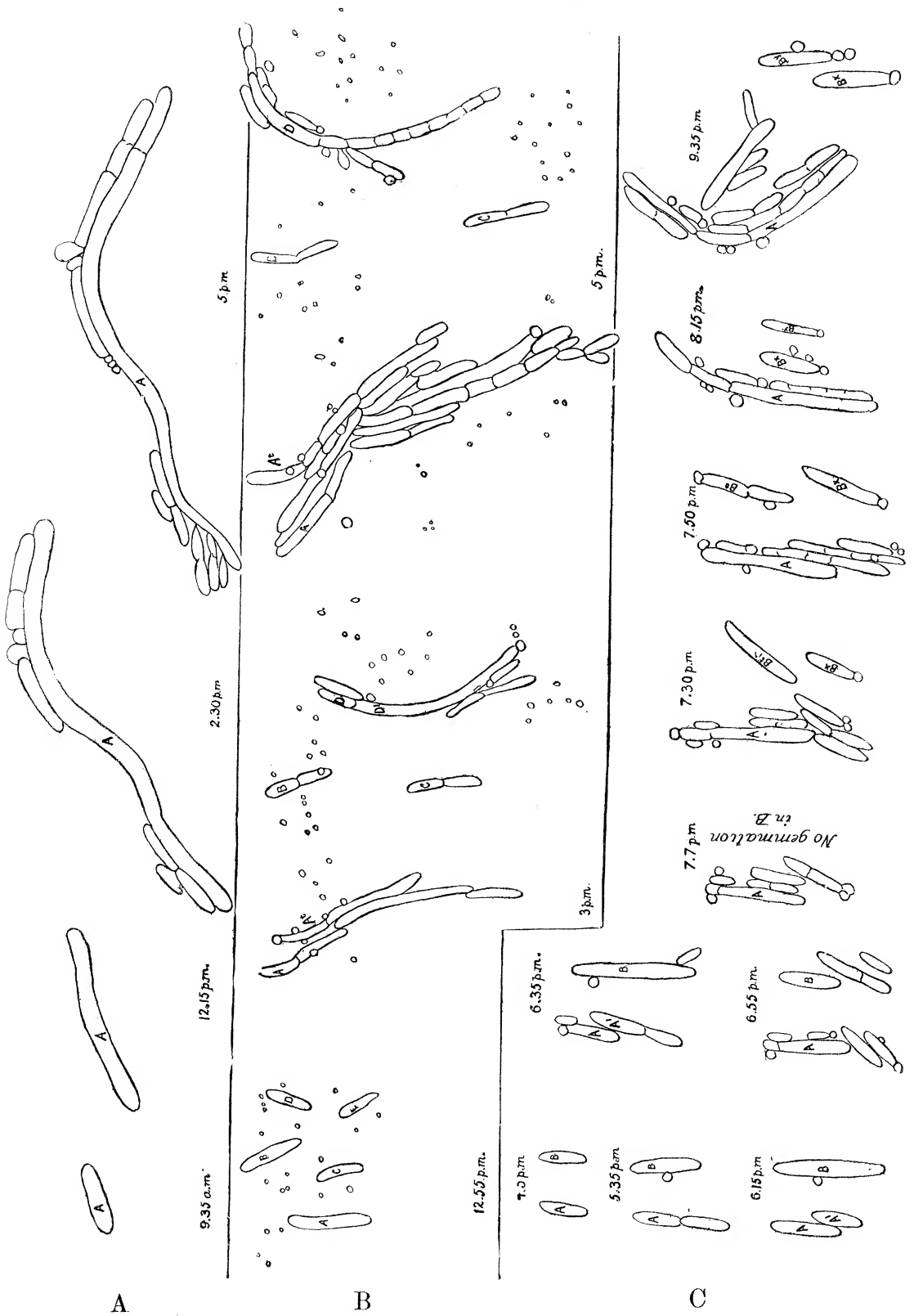
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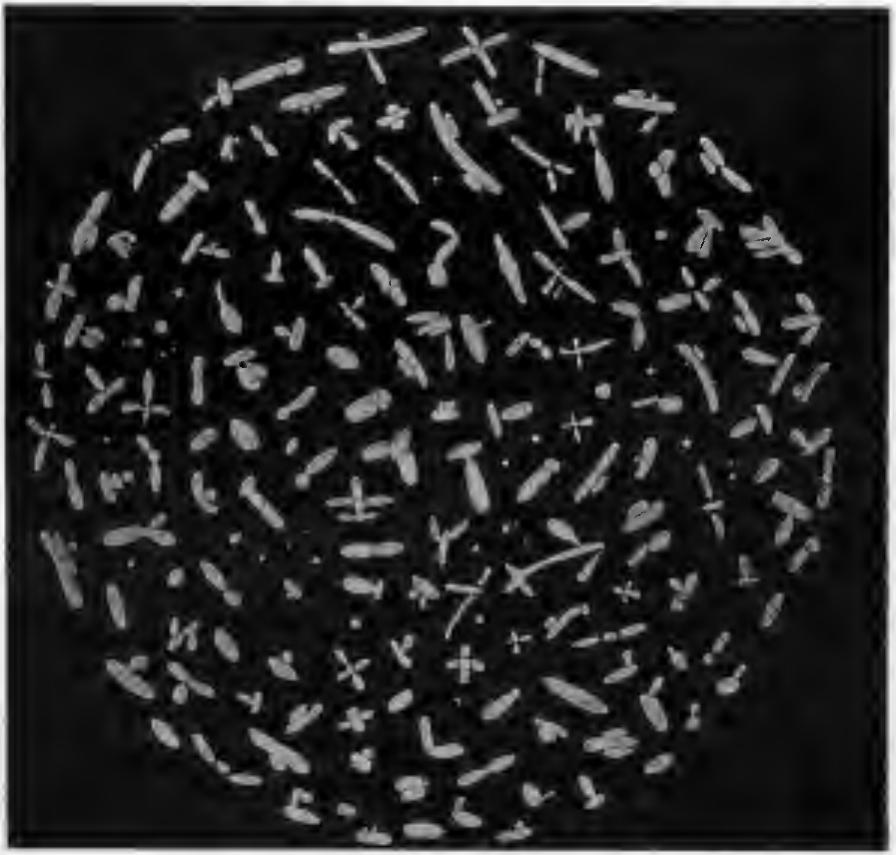


FIG. 1.—*B. typhosus.* +60 to phen. broth culture mixed with +10 to phen. broth culture from same.

FIG. 2.—*B. coli communis.* Acid broth +20 to phen. mixed with broth sub-culture from same.

FIG. 3.—*B. Shiga-Kruse.* Acid broth +10 to phen. mixed with broth sub-culture from same.

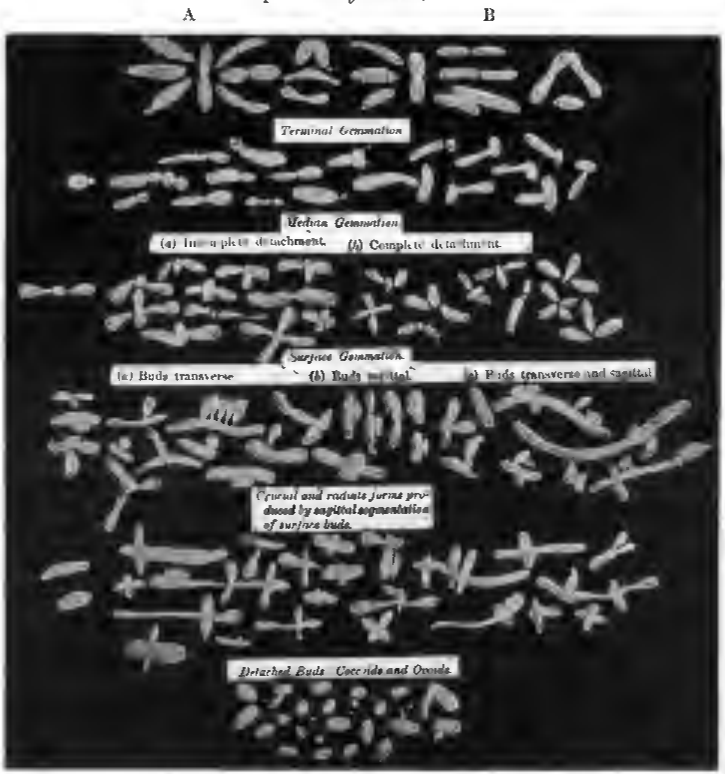
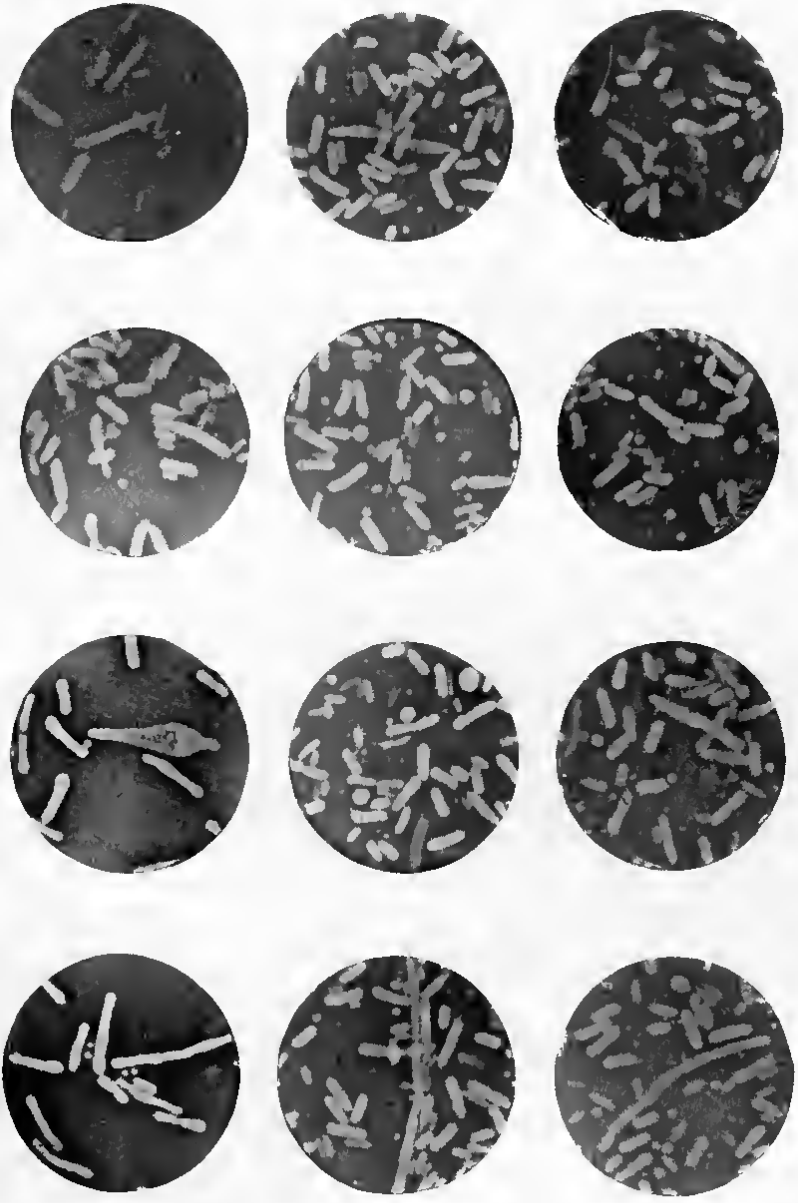
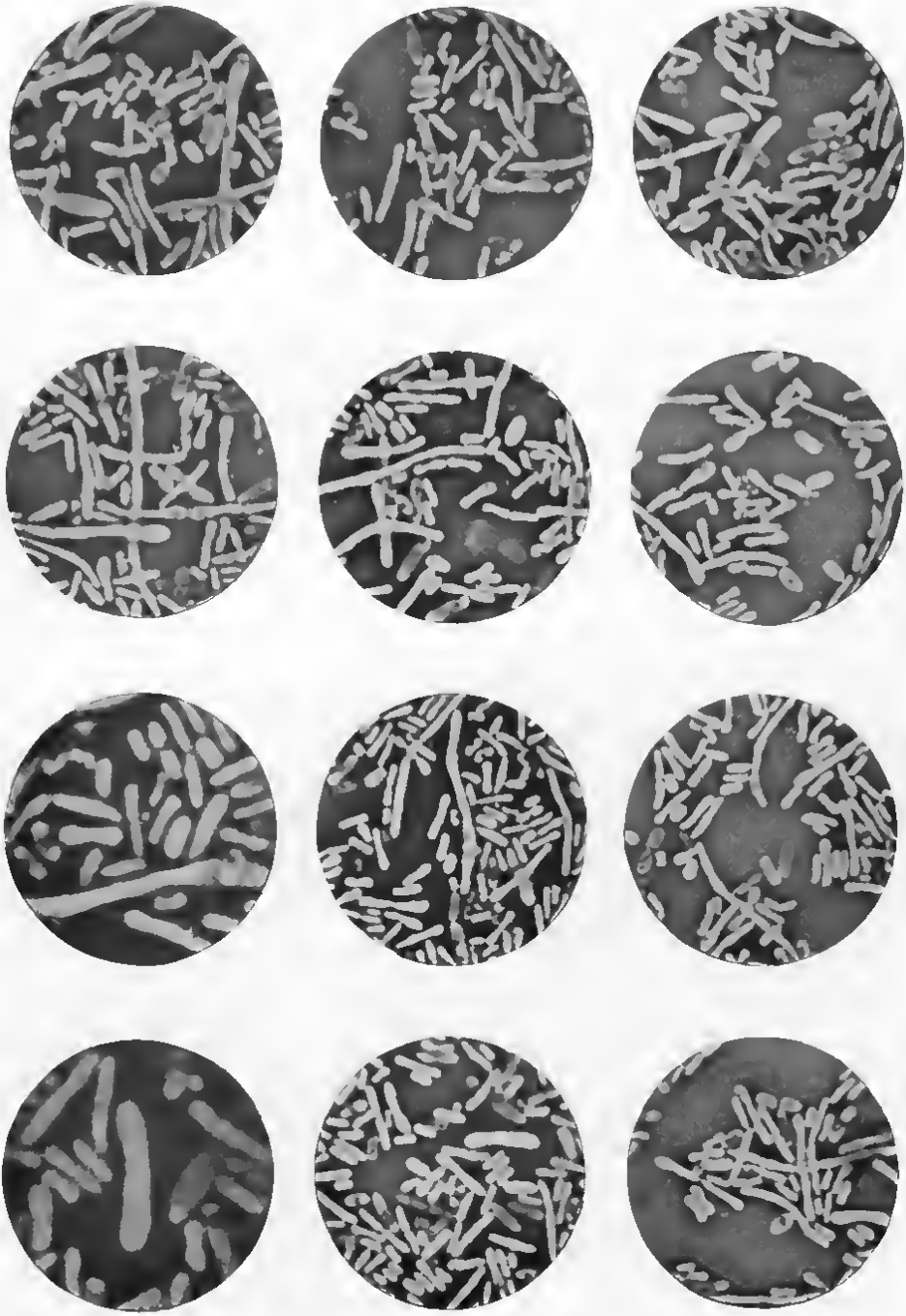


FIG. 4.—*B. Y of Hiss.* Acid broth + 20 to phen, mixed with broth sub-culture from same.



B. Shiga-Kruse.
×1500.
(Selected types.)



B. Coli Communis.
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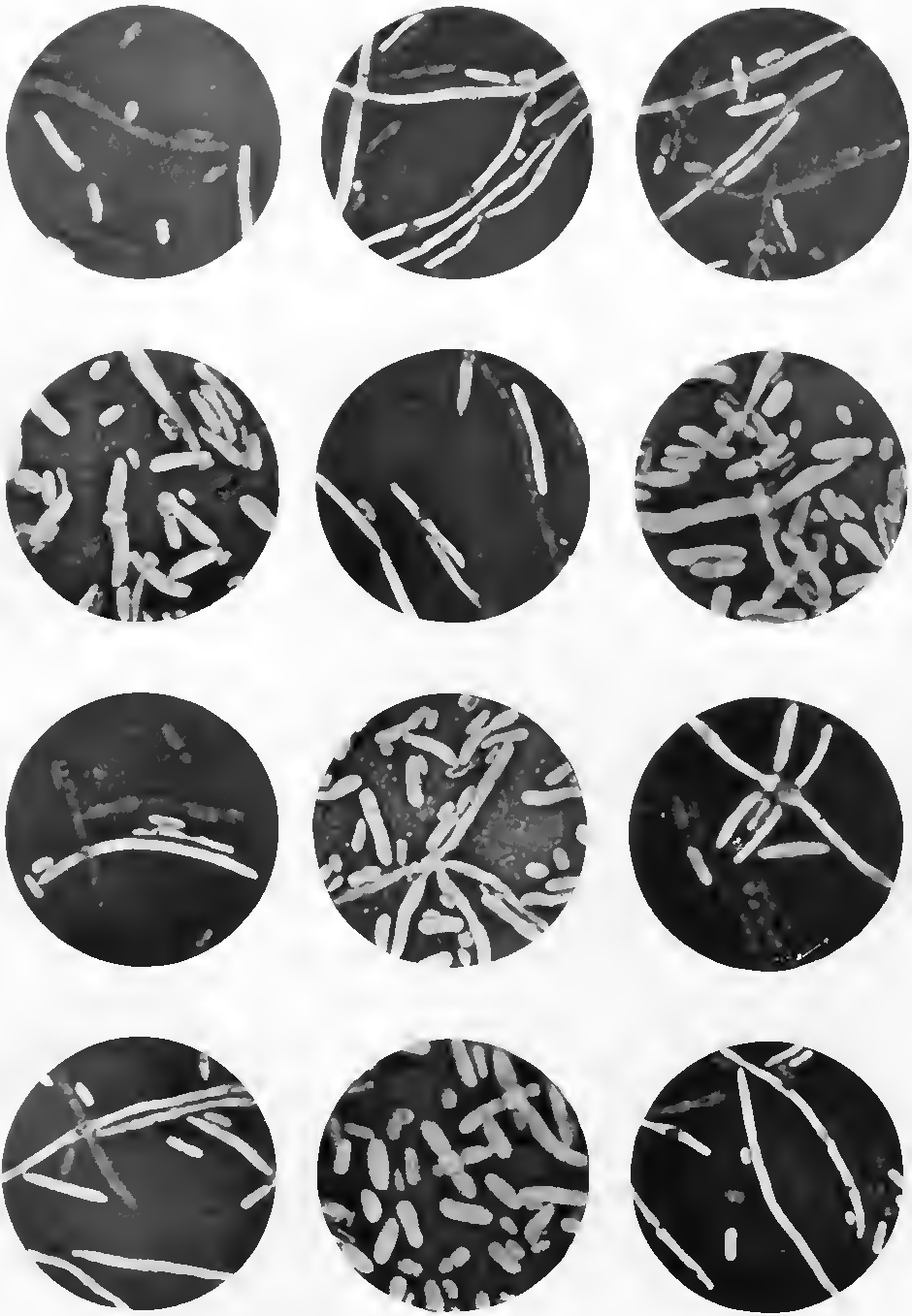

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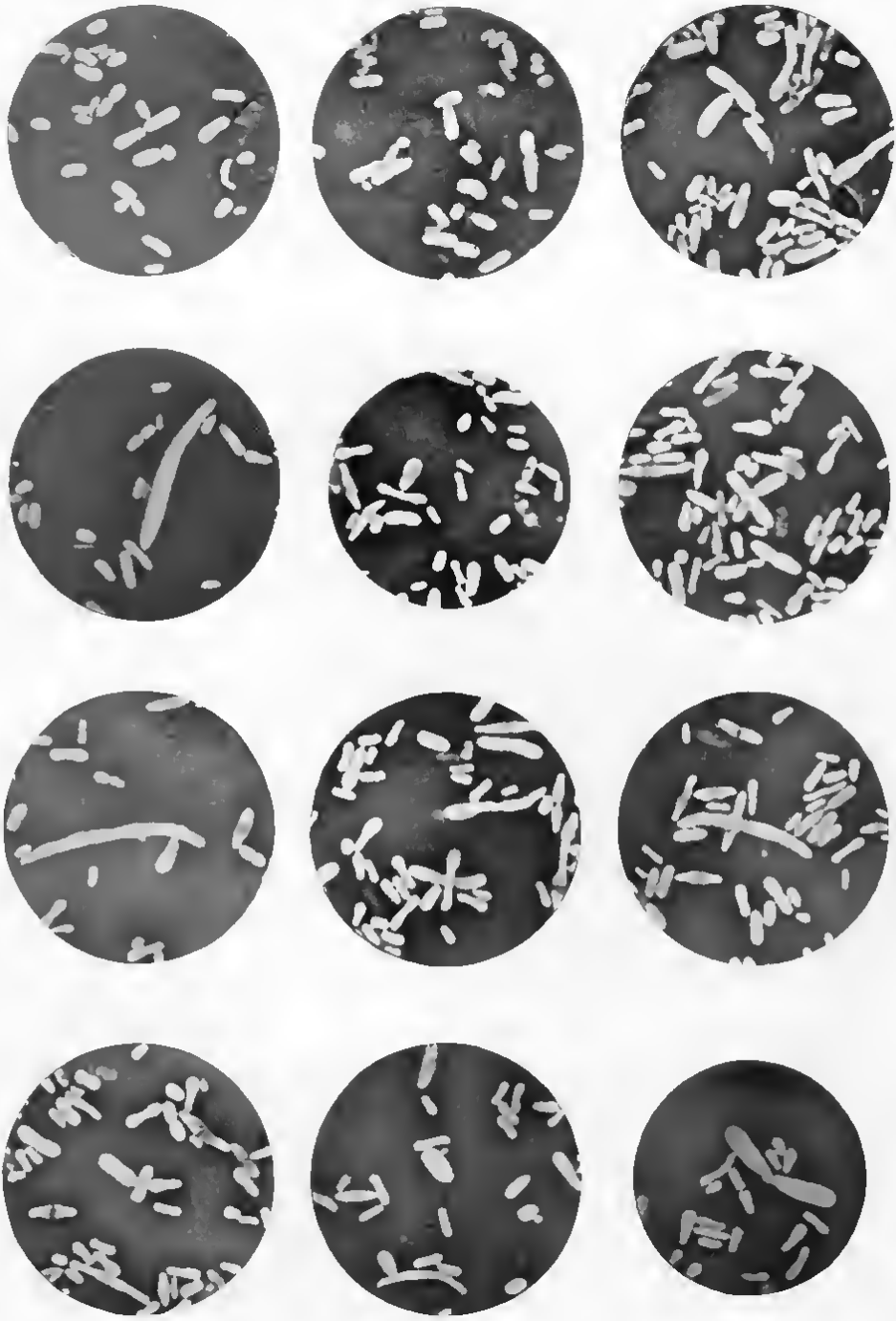

B. Y of Hiss.
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(Selected types.)